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(54) Title: REGULATION OF NITRIC OXIDE SYNTHASE ACTIVITY

(57) Abstract

This invention relates to the regulation of the activity of the enzyme nitric oxide synthase, and in particular to regulation of activity of endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS and nNOSμ). According to a first aspect, the invention provides a method of identifying modulators of AMPK–mediated activation of eNOS, comprising the step of testing putative modulators for their ability to increase or decrease phosphorylation of eNOS depending on the calmodulin and calcium ion concentrations. In an alternative aspect, the invention provides a method of identifying modulators of AMPK–mediated inhibition of eNOS, comprising the step of testing a putative modulator for its ability to decrease or increase AMPK–mediated phosphorylation of eNOS in the presence of limiting calcium ions. Preferably specific phosphorylation of threonine 495 is assessed. According to a second aspect, the invention provides a method of identifying modulators that either promote or inhibit phosphorylation of nNOS and nNOSμ at Ser–1417. Compounds which activate the AMP–activated protein kinase are expected to be useful in the treatment of ischaemic heart disease by promoting both glucose and fatty acid metabolism, as well as by increasing NOS activity to improve nutrient and oxygen supply to the myocytes and to reduce mechanical activity. These compounds would also have utility in the treatment of pulmonary hypertension and in obstructive airways disease.

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REGULATION OF NITRIC OXIDE SYNTHASE ACTIVITY

This invention relates to the regulation of the activity of the enzyme nitric oxide synthase, and in particular to regulation of activity of endothelial and neuronal nitric oxide synthases. We have found that the phosphorylation of endothelial and neuronal nitric oxide synthases by several protein kinases, including protein kinase C and the AMP-activated protein kinase, regulates their activity .

BACKGROUND OF THE INVENTION

Nitric oxide (NO) has recently been recognised as an important mediator of a very wide variety of cellular functions, and is present in most if not all mammalian cells (Moncada, S. and Higgs, A., 1993). It is implicated in a range of disorders, hypertension, hypocholesterolaemia, diabetes, heart failure, aging, inflammation, and the effects of cigarette smoking, and is especially important in vascular biology. It regulates systemic blood pressure as well as vascular remodelling (Rudic et al., 1998) and angiogenesis in response to tissue ischaemia (Murohara et al., 1998). NO is synthesised from the amino acid L-arginine by the enzyme nitric oxide synthase (NOS).

Three isoforms of NOS have been identified: neuronal NOS (nNOS), which is found in neuronal tissues and skeletal muscle (nNOSμ isoform); inducible NOS (iNOS), found in a very wide variety of mammalian tissues including activated macrophages, cardiac myocytes, glial cells and vascular smooth muscle cells; and endothelial NOS (eNOS), found in vascular endothelium, cardiac myocytes and blood platelets. Endothelial cells produce NO in response to

shear stress generated by the streaming of blood on the endothelial layer.

The three isoforms of NO synthase have an amino acid sequence identity of approximately 55%, with strong sequence conservation in regions involved in catalysis. For all three isoforms, the mechanism of NO synthesis involves binding of the ubiquitous calcium regulatory protein calmodulin (CaM) to the enzyme. However, the conditions under which CaM is bound appear to be different for iNOS, at least insofar as calcium concentration is concerned. These three NOS enzymes have been intensively studied, and the field has been recently reviewed; see for example Michel and Feron (1997); Harrison (1997); and Mayer and Hellens (1997). Although it was known from earlier studies that eNOS could be multiply phosphorylated, the mechanism of these phosphorylation events, including the enzyme responsible for phosphorylation, and the role of phosphorylation in modulation of eNOS function was not known.

AMP-activated protein kinase (AMPK) is a metabolic stress-sensing protein kinase which is known to play an important role in the regulation of acetyl-CoA carboxylase, leading to the acceleration of fatty acid oxidation during vigorous exercise or ischaemia. AMPK is well known as a regulator of lipid metabolism, and in particular is known to have a role in cholesterol synthesis, as reviewed in Hardie and Carling (1997). The AMPK is also considered to play an important role in exercise-enhanced glucose transport (Hayashi *et al.*, (1998) which is distinct from the insulin-mediated glucose uptake mechanism. AMPK has mainly been studied in the liver, heart and skeletal muscle. AMPK has been purified, and the genes encoding the enzyme subunits were cloned (See International Patent Applications

numbersPCT/GB94/01093and PCT/US97/00270 and publication WO97/25341).

The mammalian AMPK (Mitchelhill *et al*, 1994) is related to the *Saccharomyces cereviseae* SNF1 protein kinase. It is required for the expression of glucose-repressed genes in response to nutritional stress which requires growth on alternative carbon sources (Celenza and Carlson, 1986); both the mammalian and yeast kinases are activated by upstream kinases (Hardie and Carling, 1997). The AMPK is involved in metabolic stress responses through phosphorylation at Ser-79 and concomitant inhibition of acetyl-CoA carboxylase and HMG-CoA reductase (Hardie and Carling, 1997). Multiple AMPK isoforms occur. They comprise $\alpha\beta\gamma$ heterotrimers consisting of either $\alpha 1$ or $\alpha 2$ catalytic sub-units (Stapleton *et al*, 1996; Stapleton *et al*, 1997a), together with the non-catalytic subunits β and γ (Mitchelhill *et al*, 1994; Carling *et al*, 1994; Stapleton *et al*, 1994), which are related to the yeast sip1p and snf4p respectively.

The AMPK $\alpha 2$ sub-unit gene is on chromosome 1 (Beri *et al*., 1994), the $\alpha 1$ sub-unit gene is on chromosome 5, the $\beta 1$ and $\gamma 1$ sub-unit genes are on chromosome 12, the $\beta 2$ sub-unit gene is on chromosome 1, and the $\gamma 2$ sub-unit gene is localised on chromosome 7 (Stapleton *et al*, 1997). A $\gamma 3$ gene has been detected using an expressed sequence tag (EST) generated by genome sequencing (Accession No AA178898).

One of the genes encoding eNOS is on chromosome 7, close to the gene for the $\gamma 2$ sub-unit of AMPK. Another gene encoding nNOS is found on chromosome 12. (The human gene map; SEE http://www.ncbi.nlm.nih.gov/cgi-

bin/SCIENCE96/tsrch?QTEXT=nitric+oxide+synthase)

Recent work has shown that the AMPK in cardiac and skeletal muscle is activated by vigorous exercise or by ischaemic stress (Winder and Hardie, 1996; Vavvas *et al*, 1997; Kudo *et al*, 1995). This led us to investigate the localization of the AMPK isoforms in these tissues. The AMPK-α2 isoform is present in capillary endothelial cells in cardiac and skeletal muscle, and the AMPK-α1 isoform occurs in cardiac myocytes and vessels. The presence of AMPK in endothelial cells led us to test bacterially-expressed eNOS as a substrate, and we found that it is readily phosphorylated by either AMPK-α1 or AMPK-α2.

We have now surprisingly found that the AMP-activated protein kinase phosphorylates and regulates endothelial NO synthase. We find that the AMPK phosphorylates eNOS at two sites. In the presence of calcium and calmodulin, Ser-1177 in the human sequence, and Ser-1179 for the bovine sequence is phosphorylated in the COOH-terminal tail of the enzyme, causing activation of eNOS by shifting the calmodulin-dose dependence. In the absence of added calcium and calmodulin, phosphorylation also occurs at Thr-495 in the eNOS calmodulin-binding sequence, and inhibits the enzyme. Ischaemia of the heart causes activation of the AMPK and of eNOS, mimicking the effects of phosphorylation at Ser-1177. Phosphopeptide-specific antibodies to phosphorylated Ser-1177 were used to confirm that this site was phosphorylated during ischaemia. Our results are of special interest because they identify a link between metabolic stress, which reduces ATP and increases AMP, and signalling through eNOS to control nutrient availability (via arterial vasodilation) as well as suppressing myocardial contraction. This couples the metabolic status of endothelial cells and myocytes with the

vascular supply and mechanical demands. Our results provide a new insight into the post-translational regulation of eNOS which is of particular significance for the cardiovascular and skeletal muscle field. In addition, similarities in structure and behaviour between eNOS and nNOS have been identified, enabling us to identify modulators of the activity of both these enzymes.

SUMMARY OF THE INVENTION

According to a first aspect, the invention provides a method of identifying modulators of AMPK-mediated activation of a nitric oxide synthase enzyme selected from the group consisting of eNOS, nNOS and nNOSμ, comprising the step of testing the ability of putative modulators to increase or decrease phosphorylation of the enzyme; said increase or decrease depending on the calmodulin and calcium ion concentrations.

Preferably the specific phosphorylation of Ser-1177 is assessed in the presence of calcium and calmodulin.

In an alternative aspect, the invention provides a method of identifying modulators of AMPK-mediated inhibition of eNOS, comprising the step of testing a putative modulator for its ability to decrease or increase AMPK-mediated phosphorylation of eNOS in the presence of limiting calcium ions. Preferably specific phosphorylation of Thr-495 is assessed.

Compounds able to increase phosphorylation of Ser-1177 or decrease phosphorylation of Thr-495 are referred to herein as activators, and compounds able to decrease phosphorylation of Ser-1177 or increase phosphorylation of Thr-495 are referred to as inhibitors.

In both aspects of the invention, one or more of the following activities may optionally be additionally assessed for each putative activator or inhibitor identified by the method of the invention:

(a) Effect on smooth muscle contraction;

(b) Effect on inotropic activity of the heart;

(c) Effect on chronotropic activity of the heart; and

(d) Effect on platelet function.

It is expected that because the phosphorylation site equivalent to Thr-495 in the eNOS calmodulin-binding site is absent from the neuronal form of NOS, inhibitors and activators identified by the method of the invention will have at least some degree of tissue specificity.

Compounds that activate the AMP-activated protein kinase are expected to be useful in ischaemic heart disease by promoting both glucose and fatty acid metabolism, as well as by increasing NOS activity to improve nutrient and oxygen supply to the myocytes and to reduce mechanical activity. These compounds would also have utility in pulmonary hypertension and in obstructive airways disease.

For the purposes of this specification it will be clearly understood that the word "comprising" means "including but not limited to", and that the word "comprises" has a corresponding meaning.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows immunofluorescence localization of AMPK-α2 in the heart and in the tibialis anterior muscle.

Panel A shows a negative control section of rat heart stained with control rabbit IgG and control mouse IgG, together with anti-rabbit-FITC and anti-mouse-Texas Red.

Panel B shows a section of rat heart stained with affinity-purified rabbit polyclonal antibody against AMPK-α2 (491-514) and anti-rabbit-FITC.

Panel C shows the same section as Panel B, stained with a monoclonal antibody against rat endothelium recA-1 and anti-mouse-Texas Red.

Panel D shows the overlay of Panels B and C. Colocalization can be seen by the coincidence of staining. The arrows highlight specific endothelial cells that are stained by both antibodies.

Panel E shows a negative control section of rat tibialis anterior muscle stained with control rabbit IgG and control mouse-IgG, together with anti-rabbit-FITC and anti-mouse-Texas Red.

Panel F shows a section stained with affinity-purified rabbit polyclonal antibody against AMPK-α2 (491-514) and anti-rabbit-FITC.

Panel G shows the same section as in Panel B, stained with a monoclonal antibody against rat endothelium recA-1 and anti-mouse-Texas Red.

Panel H shows the overlay of Panels E and F. Colocalization can be seen by the coincidence of staining.

Figure 2 illustrates phosphorylation of recombinant eNOS by AMPK.

Top panel: eNOS was incubated with rat liver AMPK-α1 and [γ-^{32}P] ATP.

Lane 1: Coomassie-stained SDS-PAGE;

Lane 2: Autoradiograph.

Lower panel: ^{32}P-tryptic phosphopeptide map of eNOS.

Figure 3 shows the effect of phosphorylation of eNOS by the AMPK with or without added Ca^{2+}-CaM. Rat heart eNOS purified by 2',5'-ADP-Sepharose affinity chromatography was phosphorylated by AMPK in the presence of 0.8 μM CaM/3.2 μM Ca^{2+} (closed circles), in the absence of Ca^{2+}-CaM (closed triangles) and without AMPK (open squares). After phosphorylation, samples were diluted and eNOS activity was measured. The lower panels show phosphopeptide maps for rat heart eNOS phosphorylated in the presence and absence of added Ca^{2+}-CaM.

Figure 4 shows the effect of ischaemia on the activities of AMPK-α1, AMPK-α2 and eNOS.

Panel A shows the results of immunoprecipitation using antibody specific for AMPK-α1 and AMPK-α2, assayed using the SAMS peptide substrate. Results shown are mean ± SEM for n=5.

Panel B shows eNOS activity measured at 500 nM CaM.

Panel C shows eNOS activities with full CaM-dose responses for a representative experiment. Ischaemia time points: 0 min (open squares), 1 min (closed diamonds), 10 min (closed circles) and 20 min (open triangles). The results of 4 replicates were the same, except that in one case the 20 min ischaemia eNOS CaM-dependence remained the same as for 10 min.

Figure 5 shows a comparison of NOS sequences. Phosphorylation site sequences for eNOS and nNOS are indicated in a schematic model of NOS. Sequences from the CaM-binding region (around the Thr-495 phosphorylation site

in eNOS) and for the COOH-terminal tail (around the Ser-1177 phosphorylation site in eNOS) are shown.

Figure 6 shows the effect of treatment of bovine aortic endothelial cells with phorbol ester (PMA) and okadaic acid on eNOS activity (upper pane) and the phosphorylation at Ser-1177 and Thr-495 (lower panel).

Figure 7 shows the effect of treatment of bovine aortic endothelial cells with 3-isobutyl-1-methylxanthine (IBMX) and calyculin A on the phosphorylation at Ser-1177 and Thr-495.

Figure 8 shows a summary illustration of the regulation of eNOS by phosphorylation at Thr-495 and Ser-1177, mediated by protein kinases PKC, AMPK and Akt. Reversal of the phosphorylation at these sites is mediated by protein phosphatases PP1 and PP2A in response to treating the cells with IBMX and PMA respectively.

Figure 9 shows the effect of a 30 second bicycle sprint exercise on nNOS phosphorylation in human muscle. The nNOS was extracted from biopsy material and probed for phosphorylation at Ser-1417 using an anti-phosphopeptide antibody. The left panel shows an immunoblot, and the right panel shows quantitative analysis of 5 individuals.

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described in detail by way of reference only to the following non-limiting examples and to the figures.

We have surprisingly found that in the presence of Ca^{2+}-calmodulin (CaM) eNOS is phosphorylated by AMPK at Ser-1177, resulting in activation, whereas phosphorylation of eNOS in the absence of Ca^{2+} occurs predominantly at Thr-495, a site in the CaM-binding sequence, resulting in

inhibition. It had previously been considered that phosphorylation was solely inhibitory. We have also found that ischaemia of the heart leads to rapid activation of both isoforms of the metabolic stress-sensing enzyme AMPK and eNOS. These data suggest that the AMPK may operate an "inside-out" signalling pathway that leads to arterial vasodilation and reduced myocardial contraction, so coupling the metabolic status of endothelial cells and myocytes with the vascular supply and mechanical activity.

Example 1 Immunofluorescence Localisation of AMPK-α2 in Heart and Skeletal Muscle

Confocal immunofluorescence microscopy using affinity-purified rabbit polyclonal antibody directed against AMPK-α2 (antibody 491-414. Staining with fluorescence-labelled anti-rabbit antibody showed that the α2 isoform is found predominantly in capillary endothelial cells in both cardiac muscle and skeletal muscle, while cardiac myocytes and blood vessels showed intense but diffuse staining for the α1 AMPK isoform. In skeletal muscle, the α2 isoform was found in endothelial cells of capillaries, and in fast-twitch muscle fibres, whereas the α1 isoform was found in Type I aerobic fibres. Localisation of AMPK-α2 in capillary endothelial cells in both cardiac and skeletal muscle is illustrated in Figure 1.

Example 2 AMPK Phosphorylates Recombinant eNOS

Bacterially expressed eNOS, coexpressed with CaM by the method of Rodriguez-Crespo *et al* (1996), was phosphorylated by either AMPK-α1, as shown in Figure 2 top

panel, or AMPK-α2. Recombinant eNOS phosphorylation by immunoprecipitated AMPK-α2 was detected. Since we have been unable to purify high specific activity AMPK-α2, no further characterisation of eNOS regulation or the sites of phosphorylation by the α2 isoform was undertaken. Analysis of the phosphorylation sites in eNOS following tryptic digestion revealed four phosphopeptides generated from three separate sites (Figure 2 bottom panel, A, A', B, C). Identification of phosphorylation sites by mass spectrometry and Edman sequencing, using the modified method described by (Mitchelhill and Kemp, 1999), revealed that Ser-1177 was the most prominent phosphorylation site, as shown in Figure 2 bottom panel, A, A', and that its phosphorylation was dependent on the presence of Ca^{2+}-CaM.

Phosphopeptide isolation from in-gel tryptic digests was carried out as described by Mitchelhill *et al* (1997a). Greater than 98% of the radioactivity was recovered from the gel. Peptides isolated and characterized by mass spectrometry and Edman sequencing are set out in Table 1.

Table 1

Phosphopeptides Isolated from In-Gel tryptic Digests

Observed Mass	Phosphopeptide	Sequence	Calculated Mass
1440.0	B	KKTFKEVANAVK	1361.1(*1441.7)
1174.1	A	TQXFSLQER	1094.5(*1174.5)
1445.6	A'	IRTQXFSLQER	1363.7(*1443.7)
1176.7	C	pcLGSLVFPR	1095.6(*1175.6)

where: "pc" denotes pyridylethyl cysteine.

• denotes calculated mass of mono-phosphorylated peptide.

The location of the phosphorylation site in peptide A, TQXFSLQER, was identified by ^{32}P-phosphate release sequencing (Mitchelhill *et al*, 1997a). eNOS phosphorylated by the AMPK-α1 was no longer recognized by the antibody to the eNOS COOH-terminal tail; nor was it eluted from the ADP-Sepharose affinity column by 100 mM NADPH. These properties prevented the direct confirmation of Ser-1177 phosphorylation *in situ*. This is illustrated in Venema *et al*, 1996.

A second site, Thr-495, was phosphorylated in the absence of Ca^{2+}-CaM or when EGTA was present. This is illustrated in Figure 2 bottom panel, B. This residue is located in the CaM-binding sequence,

TRKKT^{495}FKEVANAVKISASLM,

between the oxidase and reductase domains of eNOS (Venema *et al*, 1996). Ser-101 in the N-terminal region of eNOS was identified as a minor site of phosphorylation (Figure 2 bottom panel, C).

Synthetic peptides containing Thr-495 or Ser-1177 were readily phosphorylated by AMPK, with similar kinetic values to the SAMS peptide substrate. The peptide containing Thr-495, GTGITRKKTFKEVANAVK, was phosphorylated with a Km of 39 ± 10 μM and a Vmax of 6.7 ± 0.6 μmol/min/mg, whereas the peptide containing Ser-1177, RIRTQSFSLQERQLRG was phosphorylated with a Km of 54 ± 6 μM and a Vmax of 5.8 ± 0.3 μmol/min/mg. These are comparable to results obtained using the well-characterized SAMS peptide substrate, which has a Km 33 ± 3 μM and a Vmax of 8.1 ± 1.5 μmol/min/mg (Michell *et al*, 1996). The *in vitro* phosphorylation of the peptides confirms the identification sites of phosphorylation.

Example 3 Effect of Ca^{2+}-CaM on Phosphorylation of eNOS by AMPK

The eNOS activity was determined by measuring L-[^{3}H]-citrulline production, using the method of Balligand *et al*, 1995. The recombinant eNOS was coexpressed with CaM, as described by Rodriguez-Crespo and Ortiz de Montellano, 1996. Partially purified rat heart eNOS contained some Ca^{2+}-CaM. In the absence of added EGTA, CaM dependence was observed at 0-100 nM added CaM. In order to investigate the changes in NOS activity with phosphorylation in the absence and presence of Ca^{2+}-CaM, EGTA buffering was used to achieve CaM dose response curves in the range 0-1 µM. Routinely, 7-15 µM EGTA was added to make eNOS activity dependent upon added CaM. Where Ca^{2+}-CaM was used in the phosphorylation reaction prior to eNOS assay, the samples were either diluted so that the extra Ca^{2+}-CaM was negligible, or the indicated concentrations represent total final concentrations of added Ca^{2+}-CaM.

Cardiac eNOS was partially purified as follows. Twenty rat hearts were homogenised in 80 ml of ice-cold buffer A [50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM NaF, 5 mM Na Pyrophosphate, 10 µg/ml Trypsin inhibitor, 2 µg/ml Aprotinin, 1 mM Benzamidine, 1 mM PMSF, 10% Glycerol, 1% Triton-X-100]. The homogenate was put on ice for 30 min and centrifuged at 16,000 x g for 30 min. The supernatant was incubated with 2 ml of 2',5'-ADP-Sepharose (Bredt and Snyder, 1990). The suspension was incubated for one hour before washing in a fritted column, with 20 ml of buffer A and 20 ml of buffer A containing 0.5 M NaCl, and then with 20 ml of buffer B [50 mM Tris-HCl, pH 7.5, 1 mM DTT, 10% Glycerol, 0.1% Triton-X-100]. eNOS was eluted with buffer B containing 2 mM NADPH, then subjected to

centrifugal filtration (ULTRAFREE-MC MILLIPORE) to remove NADPH. Immunoblotting was used for selective detection of eNOS rather than nNOS.

Phosphorylation of eNOS by AMPK in the presence of Ca^{2+}-CaM resulted in activation, but CaM-dependence was retained, as shown in Figure 3 top panel. Activation shifted the dose response curve for CaM to the left. Phosphopeptide mapping revealed that activation of eNOS was correlated with phosphorylation of Ser-1177 but not of Thr-495, as shown in Figure 3 lower panel. Phosphorylation without added Ca^{2+}-CaM enhanced Thr-495 phosphorylation, suppressed Ser-1177 phosphorylation, and inhibited eNOS activity (Figure 3 top panel). The inhibition of eNOS activity by Thr-495 phosphorylation is consistent with earlier reports that phosphorylation of synthetic peptides corresponding to this region by protein kinase C inhibits CaM-binding (Matsubara *et al*, 1996). Similar results have been reported for nNOS (Loche *et al*, 1997).

Example 4 Effect of Ischaemia on Activities of AMPK-α1, AMPK-α2 and eNOS

Langendorf preparations of isolated perfused rat heart were subjected to ischaemia according to the method of Kudo *et al* (1995). AMPK-α1 and AMPK-α2 isoforms were immunoprecipitated using α2 (490-516) or α1 (231-251) antibodies, and assayed using the SAMS peptide substrate (Michell *et al*, 1996; Hardie and Carling, 1997). eNOS activity was measured as described in Example 3. The results are shown in Figure 4. Both α1 and α2 isoforms are activated, as shown in Figure 4A, indicating that AMPK is activated in both capillary endothelial cells, which have predominantly the α2 isoform, and in cardiac myocytes,

which have predominantly the α1 isoform. AMPK activation during ischaemia is also accompanied by eNOS activation and changes in the CaM dependence, as shown in Figures 4B and 4C, mimicking the effect of eNOS phosphorylation by AMPK *in vitro*, as shown in Figure 3.

Polyclonal antibodies were raised against synthetic phosphopeptides based on the eNOS sequence: RIRTQSpFSLQER and GITRKKTpFKEVANCV. Rabbits were immunized with phosphopeptides coupled to keyhole limpet haemocyanin and then emulsified in Freund's complete adjuvant, using conventional methods. The antibodies were purified using the corresponding phosphopeptide affinity columns after thorough preclearing with dephosphopeptide affinity columns. The specificity of the purified antibodies was confirmed using both EIA and immunoblotting, confirming that they did not recognize recombinant dephospho-eNOS.

Using the anti-phosphopeptide antibodies to Ser-1177 and Thr-495 phosphorylation sites we observed that phosphorylation of Ser-1177 was increased approximately 3-fold by ischaemia, but that there was no detectable change in the Thr-495 phosphorylation under these conditions. Heart muscle contains eNOS in both capillary endothelial cells and cardiac myocytes (Balligand *et al*, 1995), with low levels of the nNOS μ isoform (Silvagno *et al*, 1996).

The sequences of the three types of NOS are compared in Figure 5, which shows the CaM-binding region and the C-terminal tail. In nNOS Ser-1417 corresponds to eNOS Ser-1177, whereas iNOS is truncated, and has a Glu in this region. Both iNOS and nNOS lack a phosphorylatable residue equivalent to Thr-495 in the CaM-binding region.

Example 5 Effect of Stimulation of Protein Kinase C on eNOS Phosphorylation

Bovine aortic endothelial cells cultured in 0.1% foetal calf serum for 20 hours (serum starved) were subjected to treatment with the protein kinase C activator 0.1 μM phorbol-12-myristate-13-acetate (PMA) for 5 min. PMA treatment increased the phosphorylation of eNOS at Thr-495 and decreased the phosphorylation at Ser-1177, as measured using anti-phosphopeptide specific antibodies. The antibodies used were the same as those described in Example 4. The results are shown in Figure 6. In cells cultured in medium without calcium we observed a 4-fold decrease in Ser-1177 phosphorylation. Furthermore, when cells were incubated in standard medium containing calcium addition of the calcium ionophore A23187 (10μM for 90 seconds) increased Ser-1177 phosphorylation by a further 7-fold. Preincubation of the cells with 0.5 μM okadaic acid prevented the dephosphorylation of Ser-1177 by PMA treatment, and greatly augmented the phosphorylation of Thr-495 (Results mean ± SEM, n =6). Since okadaic acid inhibits protein phosphatase PP2A, the results indicate that PP2A is responsible for dephosphorylation of Ser-1177. The changes observed in Thr-495 and Ser-1177 phosphorylation in response to treatment with PMA and okadaic acid were reflected in the activity of eNOS. Increased phosphorylation of Thr-495 with PMA or PMA plus okadaic acid was associated with reduced eNOS activity. Okadaic acid alone increased Ser-1177 phosphorylation without altering Thr-495 phosphorylation, and was associated with increased eNOS activity (Figure 6 upper panel).

Example 6 Effect of Inhibition of Phosphodiesterase and Phosphatase on the Phosphorylation of eNOS

The experimental details were similar to those for Example 5. Bovine aortic endothelial cells were preincubated with or without 10 nM of the phosphatase inhibitor calyculin A for 10 min, and then incubated with or without 0.5 mM of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) for 5 min. As shown in Figure 7, IBMX treatment caused enhanced phosphorylation of Ser-1177 and dephosphorylation of Thr-495. Preincubation with calyculin A prevented the dephosphorylation of Thr-495. (Results mean ± SEM, n =6). Since calyculin A inhibits protein phosphatase PP1, the results indicate that PP1 is responsible for dephosphorylation of Thr-495.

DISCUSSION

Since the identification of the Ser-1177 phosphorylation site by the present inventors, it has been recognized that other protein kinases phosphorylate at this site. In particular, the protein kinase Akt (also named PKB) phosphorylates Ser-1177 in response to stimulation of endothelial cells by vascular endothelial growth factor (VEGF)(Fulton et al.1999; Michell et al.,1999) or to fluid shear stress (Dimmeler et al., 1999; Gallis et al., 1999). In the study by Gallis et al. (1999) it was reported that fluid shear stress stimulated the phosphorylation of Ser-116 in the sequence KLQTRPSPGPPPA. Neither the kinase responsible nor the functional effects of phosphorylation of this site on eNOS has yet been identified. This phosphorylation site is present in the oxidase domain.

We have found that phosphorylation of eNOS at Thr-495 by protein kinase C occurs in endothelial cells that have been serum starved and incubated in calcium-free medium in the presence of the phorbol ester PMA. There is a reciprocal relationship between phosphorylation at Ser-1177 and Thr-495 in endothelial cells. Protein kinase C phosphorylates both sites *in vitro*, but stimulation of protein kinase C in endothelial cells with phorbol ester causes enhanced Thr-495phosphorylation but marked phosphorylation of Ser-1177. The dephosphorylation of Ser-1177 is prevented by okadaic acid but not by calyculin A, indicating that phosphatase PP2A is responsible. Okadaic acid also greatly enhances the phosphorylation of Thr-495 in response to phorbol ester. Thrombin, which also acts via protein kinase C, stimulates phosphorylation of Thr-495 and dephosphorylation of Ser-1177.

In contrast, treatment of endothelial cells with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) causes a pronounced dephosphorylation of eNOS at Thr-495 and enhanced Ser-1177 phosphorylation. Dephosphorylation of Thr-495 in response to IBMX is blocked by treatment with calyculin A, suggesting that phosphatase PP1 is responsible for Thr-495 dephosphorylation.

These relationships are summarised in Figure 8. We find that exercise of skeletal muscle results in the phosphorylation of nNOSμ at Ser-1417, the site corresponding to Ser-1177 in eNOS (see Figure 5). Electrical stimulation of rat extensor digitorum longus (EDL) muscle was found to activate the AMPK, to phosphorylate acetyl CoA carboxylase at Ser-79 (the inhibitory site), and to phosphorylate nNOSμ at Ser 1417. Similarly, in biopsies of human skeletal muscle following vigorous exercise, such as a 30-second bicycle sprint, there is a 10-fold increase in phosphorylation on Ser-79 in

acetyl CoA carboxylase and a 7.5-fold increase in nNOSμ phosphorylation at Ser-1417 (see Figure 9).

Endothelially-derived NO has a critical role in preventing premature platelet adhesion and aggregation that leads to thrombus formation (Radomski and Moncada, 1993). There is evidence that the protective effects of elevated high-density lipoprotein (HDL) on the cardiovascular system may be mediated via increased platelet NO production. Apolipoprotein E, a component of HDL, acts on a receptor (apoER2) present in platelets to stimulate the NO signal transduction pathway (Riddell *et* al., 1997; Riddell and Owen, 1999).

Activation of eNOS by phosphorylation of its COOH-terminal tail gives new insight into eNOS autoinhibition. The increased activity and shift in the CaM-dose dependence with phosphorylation at Ser-1177 suggest that in eNOS, and perhaps nNOS, the COOH-terminal tails act as partial autoregulatory sequences analogous to those in the CaM-dependent protein kinases (Kemp and Pearson, 1991; Kobe *et al*, 1996).

The COOH-terminal tail of eNOS is only fully accessible to the AMPK when Ca^{2+}-CaM is bound, consistent with this region being buried in the absence of CaM. As can be seen from Figure 5, there is a high level of similarity between eNOS and nNOS in their COOH-terminal tails, whereas iNOS is distinct. It is known that the iNOS CaM-binding, which is characterised by a low Ca^{2+}-dependence, requires both the canonical CaM-binding sequence and distal residues in the COOH-terminus that cannot be satisfied by nNOS chimeras (Ruan *et al*, 1996). Without wishing to be bound by any proposed mechanism, we believe that eNOS and nNOS are autoinhibited by their COOH-terminal tails, requiring a two-stage activation

process for full activity with both CaM-binding and phosphorylation in the tail, whereas iNOS requires only CaM binding. Recently Salerno *et al* (1997) proposed that an insert sequence in the FMN-binding domain may also be important in autoregulation.

Previous studies have shown that eNOS may be phosphorylated both *in vitro* and *in vivo*, but the precise sites of phosphorylation and the function of the phosphorylation events have not hitherto been fully characterized (reviewed in Michel and Feron, 1997). eNOS is the first example of an enzyme activated by AMPK to be identified, and is also unusual because phosphorylation can lead to either activation or inhibition, depending on the availability of Ca^{2+}-CaM. Other enzymes, notably the cyclin-dependent protein kinases, are activated or inhibited by phosphorylation, but this is catalysed by different protein kinases. Protein kinase C phosphorylates Thr-495 in eNOS, demonstrating intersecting regulatory pathways acting on eNOS by phosphorylation of Thr-495 or Ser-1177. It is also possible that persistent activation of protein kinase C, for example in response to hyperglycaemia induced by diabetes, could chronically suppress phosphorylation of eNOS at Ser-1177, and thereby reduce its activity.

The regulation of eNOS by AMPK extends the conceptual relationship between the yeast snf1p kinase and the AMPK. Snf1p kinase modulates the supply of glucose from the environment by secreting invertase whereas the mammalian AMPK integrates metabolic stress signalling with the control of the circulatory system. Thus intracellular metabolic stress signals within endothelial cells and myocytes can elicit improved nutrient supply and suppress mechanical activity of the muscle.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

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CLAIMS:

1. A method of identifying modulators of AMPK-mediated activation of a nitric oxide synthase enzyme selected from the group consisting of eNOS, nNOS and nNOSμ, comprising the step of testing putative modulators for their ability to increase or decrease phosphorylation of the enzyme, said increase or decrease depending on the calmodulin and calcium ion concentrations.

2. A method according to claim 1, in which the specific phosphorylation of Ser-1177 is assessed in the presence of calcium and calmodulin.

3. A method of identifying modulators of AMPK-mediated inhibition of eNOS, comprising the step of testing a putative modulator for its ability to decrease or increase AMPK-mediated phosphorylation of eNOS in the presence of limiting calcium ions.

4. A method according to claim 3, in which the specific phosphorylation of Thr-495 is assessed.

5. A method according to any one of Claims 1 to 4, in which one or more of the following activities is additionally assessed:

(a) Effect on smooth muscle contraction;
(b) Effect on inotropic activity of the heart;
(c) Effect on chronotropic activity of the heart; or
(d) Effect on platelet function.

6. A method according to any one of Claims 1 to 5, in which the modulator is an activator, as herein defined.

7. A method according to Claim 6, in which the activator promotes both glucose metabolism and fatty acid metabolism.

8. A method according to any one of Claims 1 to 5, in which the modulator is an inhibitor, as herein defined.

9. A method according to any one of Claims 3 to 8, in which the modulator acts preferentially on non-neuronal cells.

10. A method according to Claim 1 or Claim 2, in which the modulator promotes the dephosphorylation of Ser-1177 and inhibits eNOS activity.

11. A method according to Claim 3, in which the modulator promotes the dephosphorylation of Thr-495 and stimulates eNOS activity.

12. A method according to Claim 1 or Claim 2 in which the modulator promotes phosphorylation of nNOS or nNOSμ at Ser-1417.

13. A method according to Claim 1 or Claim 2 in which the modulator promotes dephosphorylation of nNOS or nNOSμ at Ser-1417.

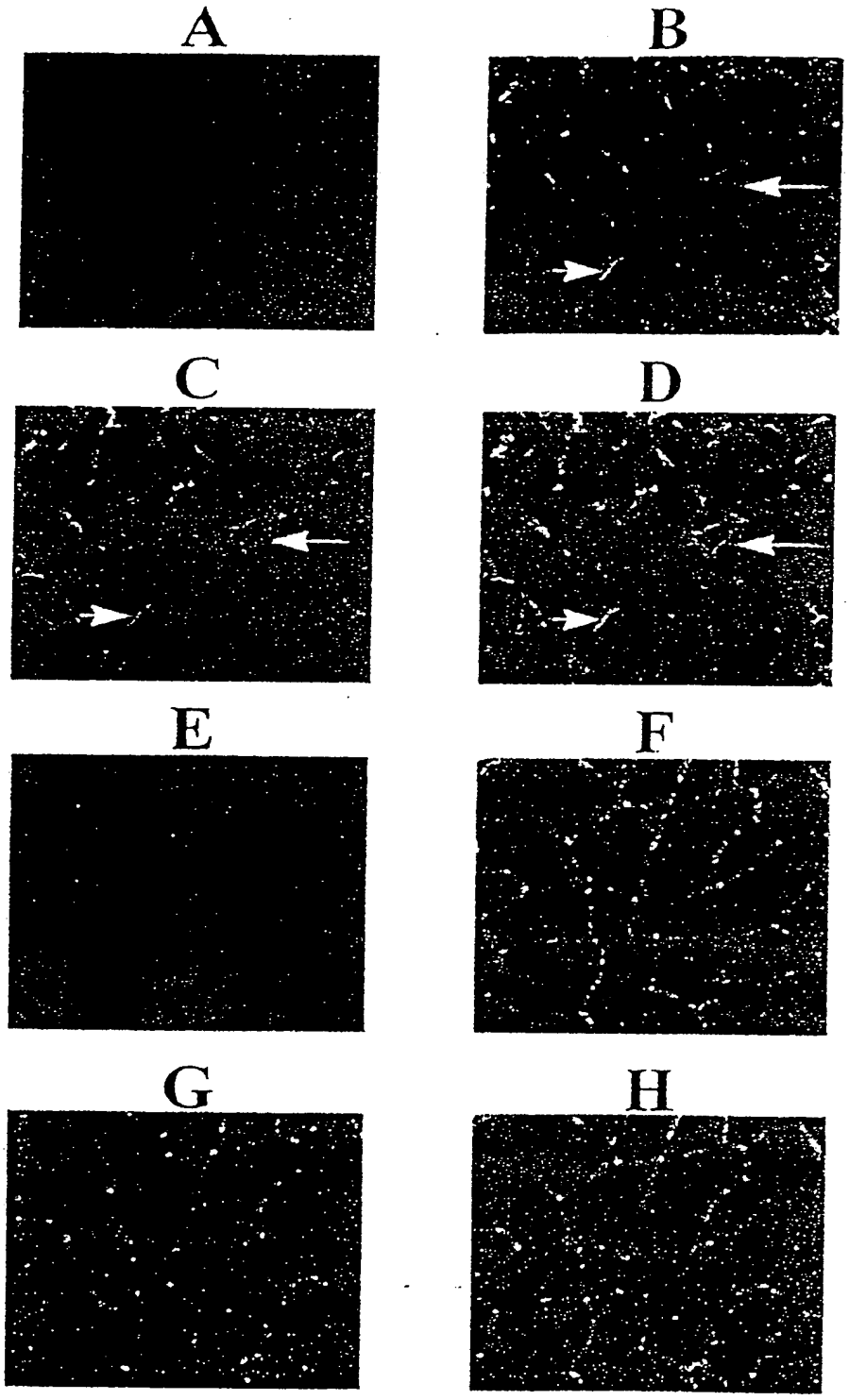
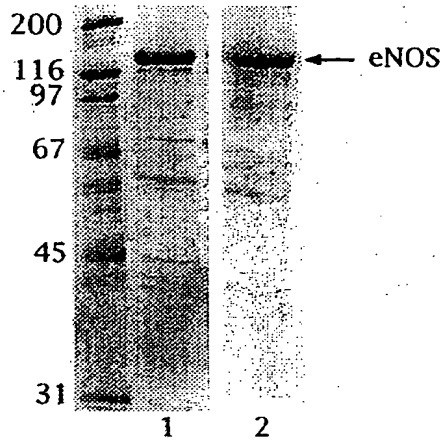


Figure 1



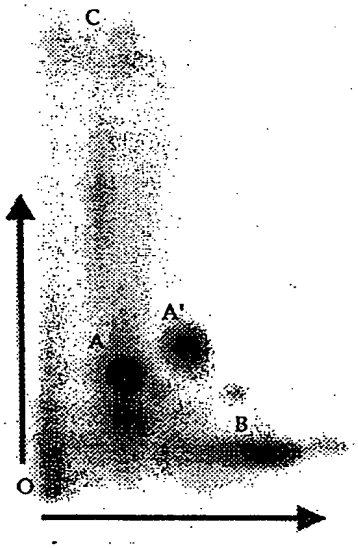
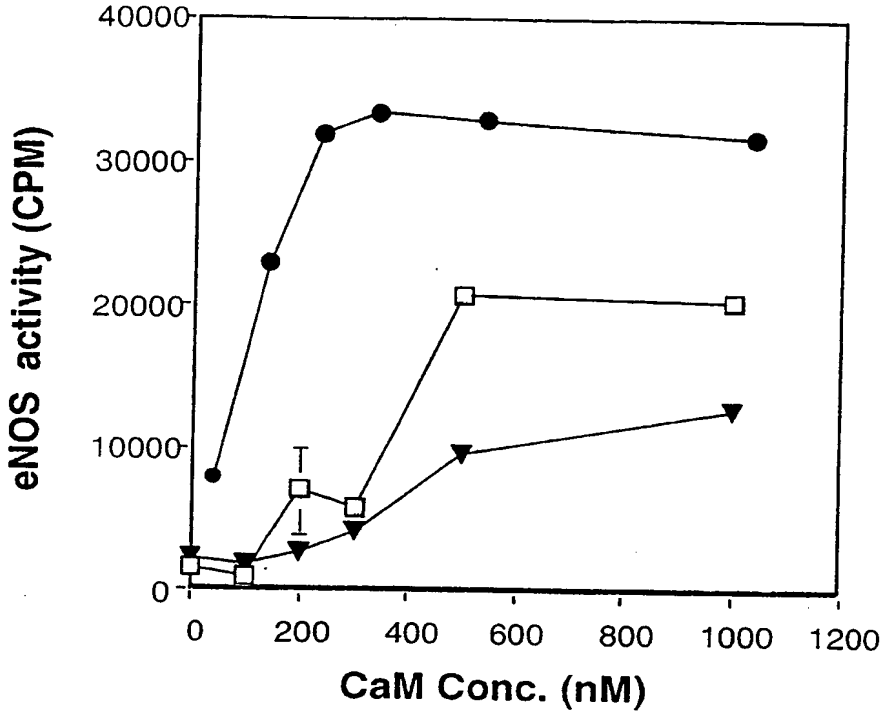


Figure 2

A


40000
30000
20000
10000
0
eNOS activity (CPM)
0
200
400
600
800
1000
1200
CaM Conc. (nM)

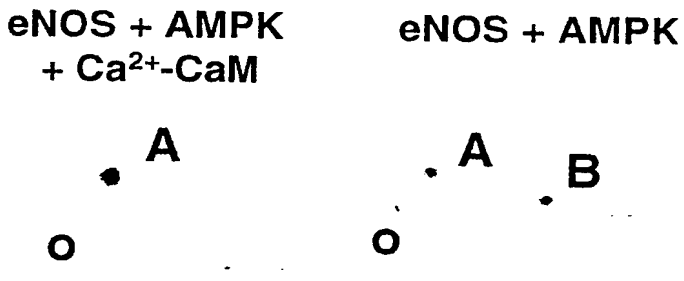
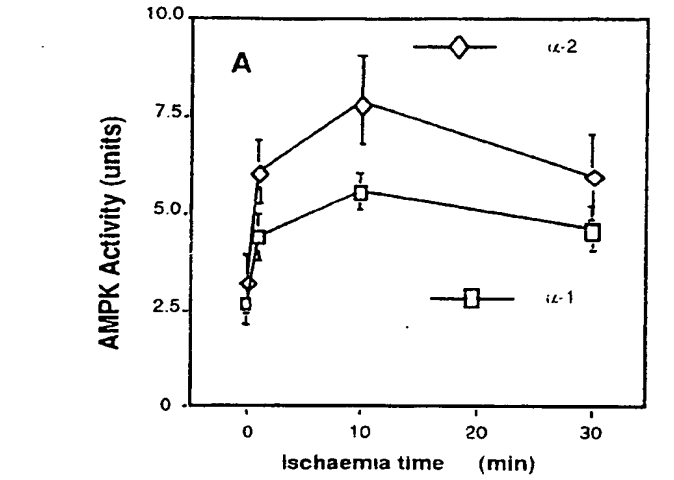
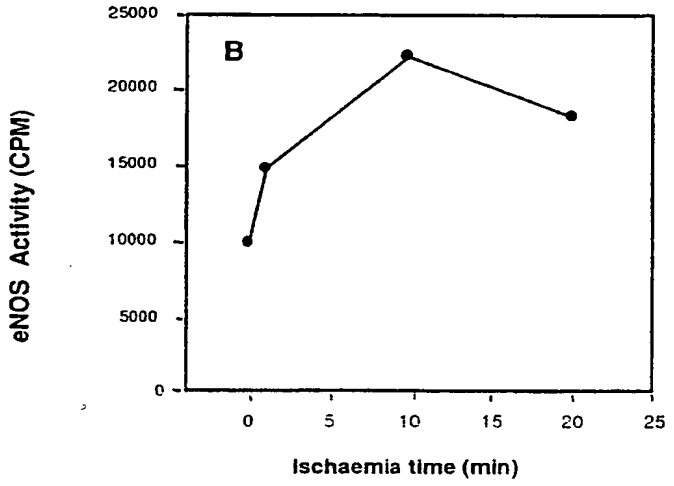

eNOS + AMPK
+ Ca2+-CaM
eNOS + AMPK
A
A
B
O
O

Figure 3


A
α-2
α-1
AMPK Activity (units)
10.0
7.5
5.0
2.5
0
0
10
20
30
Ischaemia time (min)


B
eNOS Activity (CPM)
25000
20000
15000
10000
5000
0
0
5
10
15
20
25
Ischaemia time (min)

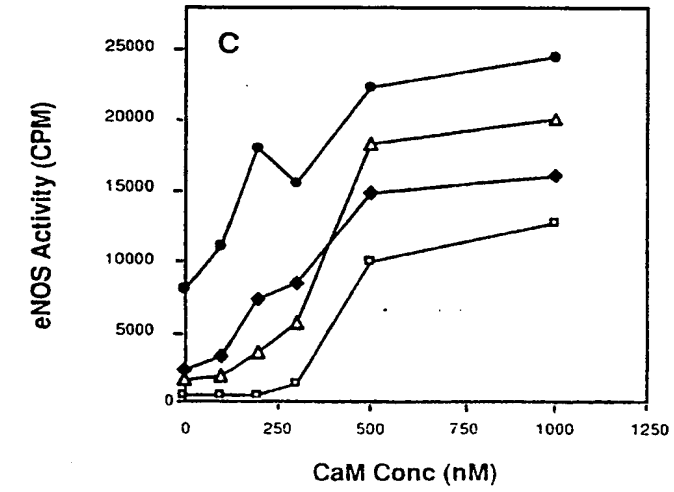

C
eNOS Activity (CPM)
25000
20000
15000
10000
5000
0
0
250
500
750
1000
1250
CaM Conc (nM)

Figure 4

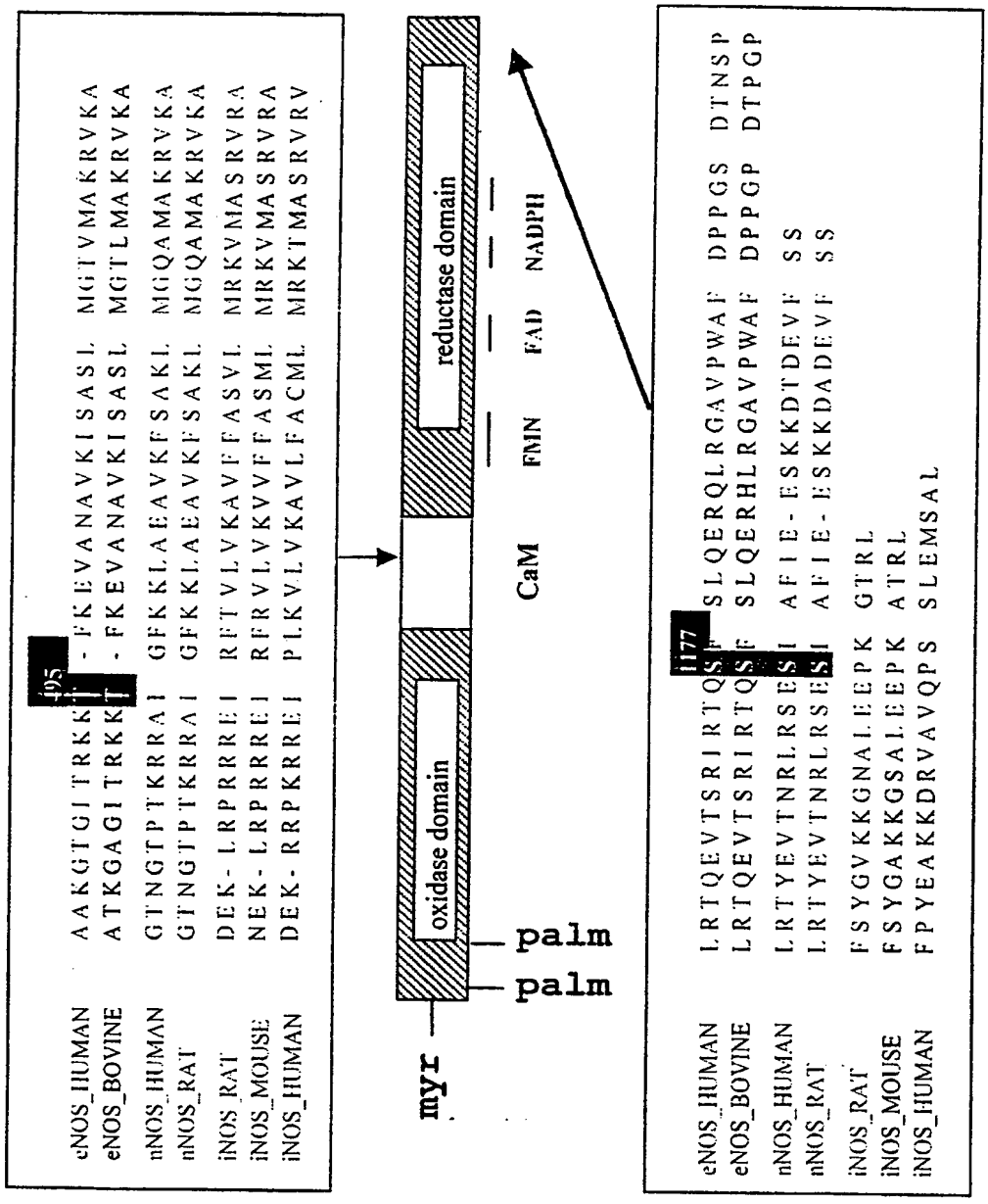

105
eNOS_HUMAN AAKGTGITRKK - FKEVANAVKISASL NGTVMAKRVKA
eNOS_BOVINE ATKGAGITRKK - FKEVANAVKISASL MGTLMAKRVKA
nNOS_HUMAN GTNGTPTKRRAI GFKKLAEAVKFSAKL MGQAMAKRVKA
nNOS_RAT GTNGTPTKRRAI GFKKLAEAVKFSAKL MGQAMAKRVKA
iNOS_RAT DEK-LRPRRREI RFTVLVKAVFFASVL MRKVMASRVRA
iNOS_MOUSE NEK-LRPRRREI RFRVLVKVVFFASML MRKVMASRVRA
iNOS_HUMAN DEK-RRPKRREI PLKVLVKAVLFACML MRKTMASRVRV
myr
oxidase domain
palm
palm
CaM
FMN
FAD
NADPH
reductase domain
1177
eNOS_HUMAN LRTQEVTSRIRTQSF SLQERQLRGAVPWAF DPPGS DTNSP
eNOS_BOVINE LRTQEVTSRIRTQSF SLQERHLRGAVPWAF DPPGP DTPGP
nNOS_HUMAN LRTYEVTNRLRSESI AFIE-ESKKDTDEVF SS
nNOS_RAT LRTYEVTNRLRSESI AFIE-ESKKDADEVF SS
iNOS_RAT FSYGVKKGNALEEPK GTRL
iNOS_MOUSE FSYGAKKGSALEEPK ATRL
iNOS_HUMAN FPYEAKKDRVAVQPS SLEMSAL

Figure 5

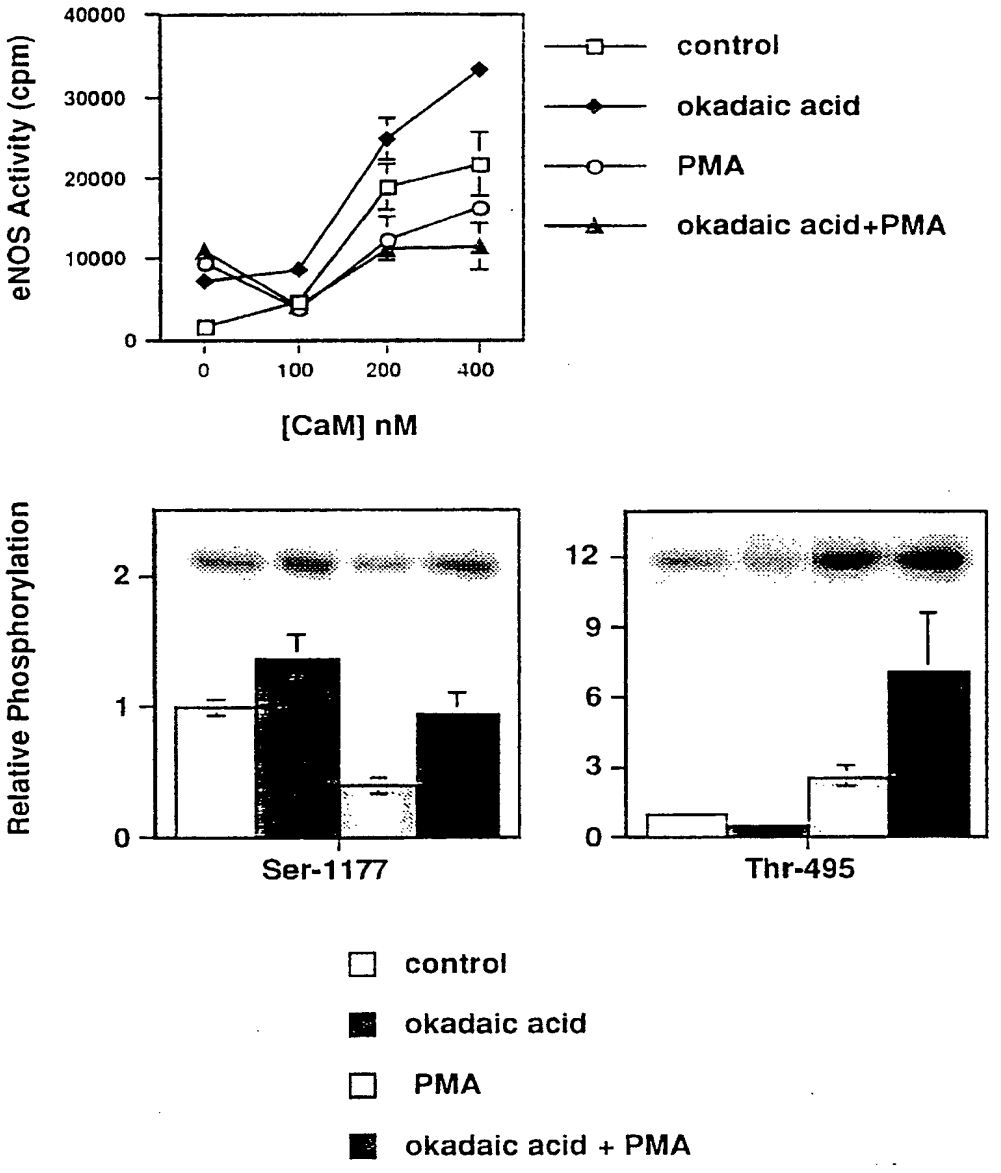

eNOS Activity (cpm)
40000
30000
20000
10000
0
0
100
200
400
[CaM] nM
control
okadaic acid
PMA
okadaic acid+PMA
Relative Phosphorylation
2
1
0
Ser-1177
12
9
6
3
0
Thr-495
control
okadaic acid
PMA
okadaic acid + PMA

Figure 6

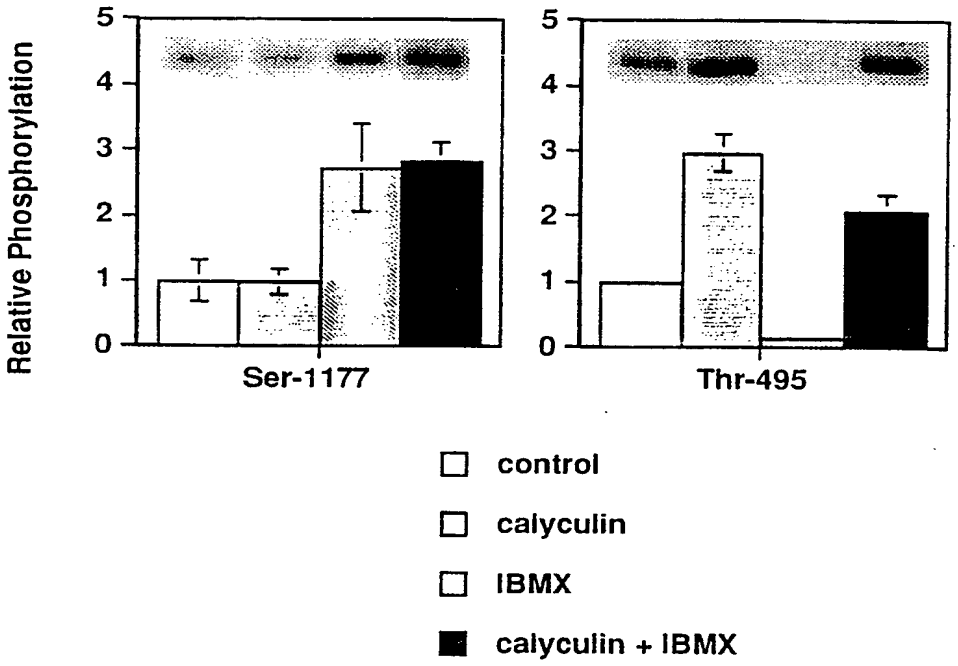

Relative Phosphorylation
5
4
3
2
1
0
Ser-1177
Thr-495
control
calyculin
IBMX
calyculin + IBMX

Figure 7

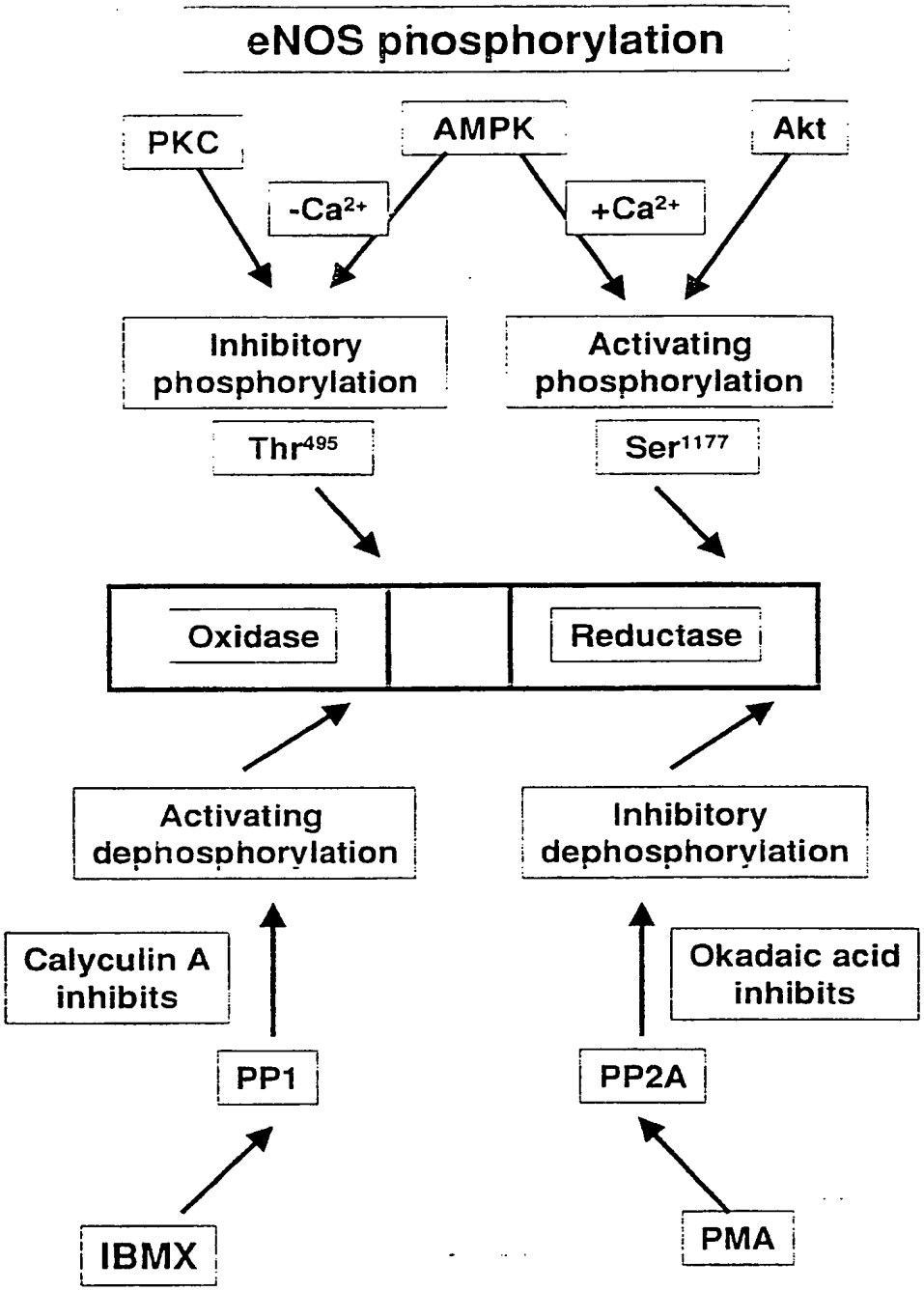

eNOS phosphorylation
PKC
AMPK
Akt
-Ca2+
+Ca2+
Inhibitory phosphorylation
Activating phosphorylation
Thr495
Ser1177
Oxidase
Reductase
Activating dephosphorylation
Inhibitory dephosphorylation
Calyculin A inhibits
Okadaic acid inhibits
PP1
PP2A
IBMX
PMA

Figure 8

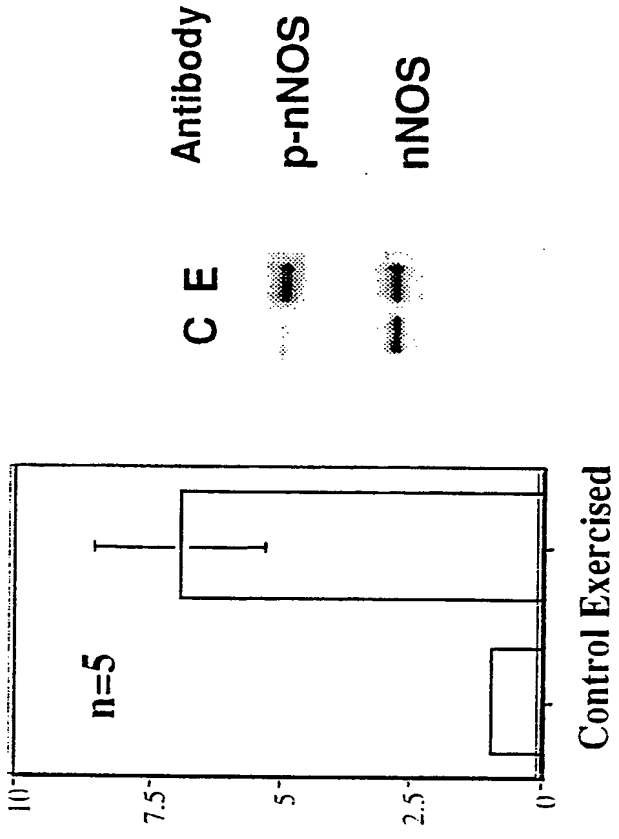

Antibody
p-nNOS
nNOS
C E
n=5
10
7.5
5
2.5
0
Control Exercised
Relative fold

Figure 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00968

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12Q 1/26, 1/27, 1/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC (WPAT - WORLD PATENTS INDEX), CHEMICAL ABSTRACTS

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
MEDLINE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, Medline, Chemical Abstracts. Search terms: nitric oxide synthase, ampk, amp protein kinase:

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	FEBS Letters, volume 443, 1999, Chen et al., "AMP-activated protein kinase phosphorylation of endothelial NO synthase", pages 285 to 289	1 to 11
P, X	WO 99/26657 (MEDICAL UNIVERSITY OF SOUTH CAROLINA) 3 June 1999	1, 3, 5 to 9
A	The Journal of Biological Chemistry, volume 267, number 16, 1992, Bredt et al., "Nitric Oxide Synthase Regulatory Sites", pages 10976 to 10981	1 to 1

☐ Further documents are listed in the continuation of Box C

☐ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 December 1999	21 DEC 1999
Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	CHRISTOPHER LUTON Telephone No.: (02) 6283 2256